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The Drosophila *beaten path* Gene Encodes a Novel Secreted Protein That Regulates Defasciculation at Motor Axon Choice Points

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Summary

In the Drosophila embryo, at specific choice points along the major motor nerves, subsets of motor axons defasciculate and then steer into their muscle target regions. Here we describe the analysis of *beaten path* (*beat*), a gene required for the selective defasciculation of motor axons at these choice points. In *beat* mutant embryos, motor axons fail to defasciculate and bypass their targets. This phenotype is suppressed by mutations in *FasII* and *conn*, two genes encoding cell adhesion molecules expressed on motor axons, suggesting that *beat* provides an antiadhesive function. *beat* encodes a novel secreted protein that is expressed by motoneurons during outgrowth. Rescue and ectopic expression experiments support the model that Beat protein is secreted by motor axons where it functions to regulate their selective defasciculation at specific choice points.

Introduction

During neuronal development, growth cones often extend along the surface of other axons, forming axon bundles or fascicles. This process of fasciculation can be highly selective; when confronted with an array of axon pathways, some growth cones display a selective affinity for specific axon fascicles (e.g., Bastiani et al., 1984; Goodman et al., 1984; Raper et al., 1984). While selective fasciculation provides a convenient highway for axon extension, and in some cases labeled pathways guide growth cones toward particular regions, growth cones must inevitably exit the main highway and steer into their specific target regions, a process called defasciculation. These events can also be highly selective, with subsets of axons leaving the main tract and forming side branches at specific choice points. A failure to defasciculate can lead growth cones to continue down the main highway, bypassing their correct targets. Which molecular mechanisms control the ability of growth cones to get on to and off of axon pathways at specific choice points, that is, to selectively fasciculate and defasciculate?

A number of cell adhesion molecules (CAMs) have been implicated in mediating axon fasciculation. The most detailed understanding has been obtained in the analysis of Fasciclin II (Fas II), a cell adhesion molecule of the immunoglobulin (Ig) superfamily in insects that is highly related to vertebrate NCAM (Bastiani et al., 1987; Harrelson and Goodman, 1988). In Drosophila, Fas II is expressed on a subset of embryonic CNS axons, many of which selectively fasciculate in three longitudinal axon pathways (Grenningloh et al., 1991; Lin et al., 1994). In *FasII* loss-of-function mutants, the axons that normally fasciculate together in these three pathways fail to do so (Lin et al., 1994). Transgenic constructs that specifically drive Fas II expression on the axons in these same three pathways can rescue the *FasII* defasciculation phenotype, creating a refasciculation of these three fascicles. Moreover, these transgenic constructs can lead to a gain-of-function phenotype in which pairs of pathways that should remain separate become abnormally joined together, and a pair of pathways that normally start together and then defasciculate into two separate pathways instead remain fasciculated as one. Ig CAMs function in axon fasciculation in vertebrates as well (e.g., Stoeckli and Landmesser, 1995).

If the expression of CAMs such as Fas II can mediate the process of selective fasciculation, then we presume that defasciculation might in part involve either the removal of these CAMs from the cell surface or the downregulation of their function. Studies in the chick have implicated polysialic acid (PSA) as a key regulator of a general form of axon defasciculation. The addition of PSA chains to the neural cell adhesion molecule NCAM is believed to decrease the ability of NCAM and other CAMs like L1/NgCAM to mediate adhesion (e.g., Rutishauser et al., 1988; Rutishauser and Landmesser, 1991).

During the development of the chick embryo, the tightly fasciculated and intermingled motor axons exit the CNS in multiple spinal nerves that converge at the plexus at the base of the limb bud where they defasciculate and sort into specific axon pathways. This occurs concomitant with a dramatic increase in the levels of PSA on motoneuron growth cones and axons. Enzymatic removal of PSA leads to an increase in the number of projection errors (Tang et al., 1992). These defects in pathfinding are likely due to an increase in NgCAM-mediated axon adhesion (Tang et al., 1994). Blocking NgCAM function in embryos in which PSA has also been removed leads to a suppression of the projection errors seen with PSA removal alone. The simplest interpretation of these results is that PSA causes a decrease in NgCAM-mediated axon fasciculation that permits motor growth cones to respond to specific (unidentified) guidance cues in the plexus.

Insights into the regulation of a more selective form of defasciculation have come from genetic studies of motor axons in the Drosophila embryo. In each abdominal hemisegment, ~50 motor axons grow into the periphery to specifically innervate 30 unique muscle targets. Motor axons exit the CNS in two distinct motor nerves: the intersegmental nerve (ISN) and the segmental nerve (SN) (Figure 1). Subsets of motor axons leave the two main motor nerves at particular choice points to form specific motor branches. The SNb and SNd branch off of the ISN in the ventral muscle region, while the main ISN continues to extend dorsally to further branch and innervate dorsal muscles. The SNc branches off of the SN in the ventral muscle region, while the main SN (called the SNa) continues to extend dorsally to further branch and innervate lateral muscles. At each

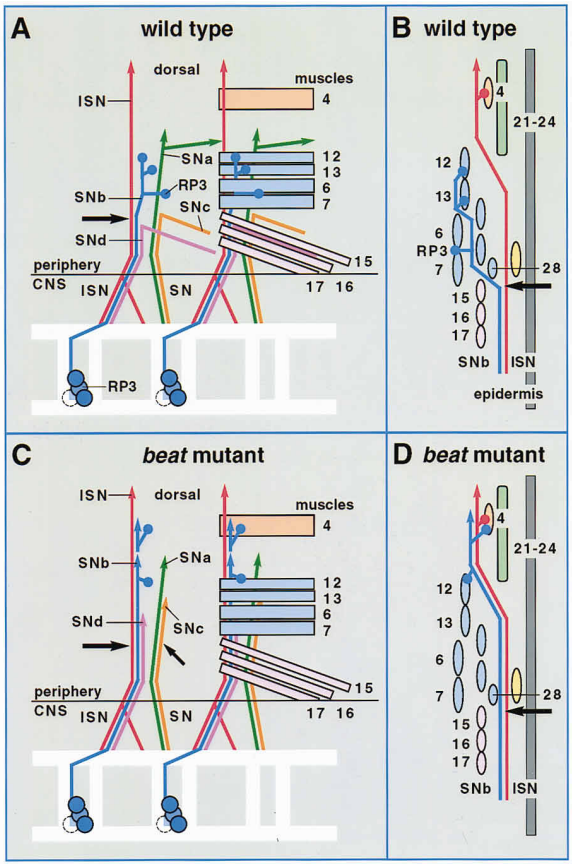


Figure 1. Motor Axon Pathways in Wild-Type and *beat* Mutant Embryos

(A) Schematic representation of 2 hemisegments (A2-A7) of a wild-type stage 17 Drosophila embryo as seen in fillet preparation. Anterior is left and dorsal is above. A subset of the musculature has been shown in the right hand segment, while the rest has been removed to reveal the motoneuron pathways: ISN (red), SNb (blue), SNd (purple), SNa (green), and SNc (orange). Muscle coloration corresponds to the color of the innervating branch: muscles 6, 7, 12, and 13 are innervated by SNb, 15, 16, and 17 by SNd, and 4 by ISN. These five branches exit the CNS in two nerve roots, ISN and SN, before branching to enter their muscle target regions in the periphery. The arrow marks the choice point where the SNb diverges from the ISN. The RP3 motoneuron, which contributes to the SNb, resides between the CNS axon commissures and projects an axon across the midline that exits the CNS in the ISN nerve root, branches in the SNb, and innervates muscles 6 and 7.

(B) Cross-sectional view showing the trajectory of the SNb after defasciculation and divergence from the ISN. SNb diverges after contacts with muscle 28, where it enters and then innervates the muscles of the ventral muscle domain (light blue), separated from the ISN by a layer of muscles.

(C) Abnormal motoneuron outgrowth in *beat* mutant embryos. Motor axons exit the CNS normally in the nerve roots, but then fail to branch and enter their muscle domains once in the periphery. The large and small arrows mark the choice points where the SNb and SNc would normally defasciculate. SNb is sometimes observed to make ectopic contacts to target and nontarget muscles by lateral sprouting from the ISN pathway.

(D) Cross-sectional view of the *beat* mutant.

branch (or choice) point, groups of motor axons rearrange their cellular contacts. They defasciculate from the bundle of motor axons in the main motor nerve, although they remain fasciculated with each other within their side branch. They then steer into their target region as they interact with several neighboring muscles. Ultimately, they defasciculate from one another and innervate their specific targets.

The motor axons of the SNb initially follow and adhere to ISN axons, but at their choice point in the ventral muscle region they defasciculate from the ISN axons and form a separate bundle which steers into the ventral muscle region. The SNb choice point has proved to be an excellent model system for the in vivo genetic dissection of guidance and steering decisions (e.g., Van Vactor et al., 1993; Lin and Goodman, 1994; Nose et al., 1994; Desai et al., 1996; Krueger et al., 1996). Six genes have been identified so far that can affect SNb guidance at this choice point.

The first gene implicated in this process is *FasII*. Fas II is normally expressed at high levels on motor axons (Van Vactor et al., 1993). Although Fas II is a potent mediator of homophilic cell adhesion, the levels of Fas II (as detected by immunocytochemistry) appear unchanged at the choice points of the motor nerves, even though different groups of axons defasciculate from the main axon pathway. When transgenic methods are used to increase the levels of Fas II on motor axons, the SNb axons fail to defasciculate at the choice point, and instead continue to extend dorsally along the ISN and fail to invade the ventral muscle region (Lin and Goodman, 1994). These studies suggest that defasciculation at the choice point requires modulation of Fas II function.

Five other genes have been identified that encode candidate regulators of Fas II function at this choice point, as loss-of-function mutations in these genes give similar SNb defasciculation phenotypes as is observed in the increased (gain-of-function) Fas II experiments. Three genes encode the receptor protein tyrosine phosphatases (RPTPs) DLAR, DPTP69D, and DPTP99A, which are expressed on motor axons (Desai et al., 1996; Krueger et al., 1996). Single mutations in the genes encoding DPTP69D and DPTP99A have no or little effect on the axons, but embryos mutant in both *Dptp69D* and *Dptp99A* show a partially penetrant version of the same phenotype as that produced by increased expression of Fas II on these same axons: the SNb fails to defasciculate from the ISN (Desai et al., 1996). Loss-of-function mutations in *Dlar* result in a similar but subtly different phenotype (Krueger et al., 1996).

Single mutations in either of two other genes—*beaten path* (*beat*; Van Vactor et al., 1993; this paper) and *sidestep* (*side*; H. Sink and C. S. Goodman, Soc. Neurosci., abstract, 1994)—result in a highly penetrant SNb defasciculation phenotype that is virtually indistinguishable from the Fas II gain-of-function phenotype: the SNb axons fail to defasciculate from the ISN and instead continue extending distally. *side* has not yet been cloned.

In this paper, we report that *beat* encodes a novel secreted protein that is expressed by motoneurons. The *beat* mutant phenotype can be rescued by expressing *beat* in motoneurons. Genetic interactions between *beat* and *FasII* (and to a lesser degree between *beat* and *connectin*, a CAM expressed on a restricted subset of motor axons; Nose et al., 1992) suggest that secretion

of Beat functions to decrease axon–axon adhesion. Misexpression of *beat* by muscles disrupts motoneuron-muscle interactions. These results suggest that Beat functions as an anti-adhesive that controls the selective defasciculation of motor axons at specific choice points.

Results

beaten path Is Required for Motor Axon Defasciculation at Nerve Branch Points

In the Drosophila embryo, motor axons exit the CNS in two nerve roots that subsequently branch into five motor nerves (described in Introduction; Van Vactor et al., 1993) (Figure 1A). Three primary branches arise from the ISN: (1) the main ISN, which innervates the dorsal muscles, (2) the SNb, which innervates the ventral longitudinal muscles, and (3) the SNd, which innervates the ventral oblique muscles. Two primary branches arise from the SN: (1) the SNa, which innervates the lateral muscles, and (2) the SNc, which innervates the ventral external muscles. The ISN and the SNa are the two routes taken by the pioneers of each nerve root.

In *beaten path* (*beat*) mutant embryos (Van Vactor et al., 1993), the two nerve roots are correctly pioneered, but at very high frequency the nerve branches fail to defasciculate and diverge at choice points. Instead, they remain fasciculated and fail to enter their target muscle domains (Figures 1C and 1D; Figure 2). For example, the SNb axons remain fasciculated with the ISN, bypassing their branch point at muscle 28 and failing to form a distinct fascicle separate from the ISN. The other nerve branches are also affected: the SNd remains fasciculated with the ISN, and the SNc remains fasciculated with the SNa. More dorsally, where the SNa typically bifurcates to form a lateral and dorsal branch, defects are also observed in a failure to bifurcate. The dorsalmost extension of the ISN is often truncated, which may indicate a defect at the site where a minor lateral subbranch of the ISN forms. Occasionally, at very low penetrance, the ISN is observed crossing the segment boundary and fasciculating with the ISN of the adjacent segment. In these cases, it appears that the ISN has failed to deadhere from the Fasciclin II–expressing m cell, a transient target for the ISN during development (Van Vactor et al., 1993) (Figure 2C). The adhesion to the m cell appears to channel the ISN into the next anterior segment, where it fasciculates with the ISN.

The common element in all of these *beat* phenotypes is that axons remain adhered to other cell surfaces from which they should disengage. The lack-of-defasciculation phenotype is remarkably similar to that observed when the cell adhesion molecule Fas II is overexpressed on motor axons (Lin and Goodman, 1994; see Introduction, this paper). This suggests that *beat* may provide an anti-adhesive function, opposing the adhesion promoted by such cell adhesion molecules as Fas II.

In *beat* mutant embryos, the ultimate destination of most of the motor axons that have failed to defasciculate is not known, but in the case of the SNb they appear to continue along the ISN, often beyond the ventral muscle domain. SNb motor axons can be seen making ectopic contact with muscle 4, a target normally innervated by the ISN (Figures 2D and 2E). The SNb axons can also make contact with muscles 12 and 13 by lateral sprouting from the common pathway (Figures 1C and 1D).

The penetrance of these defects is not 100%, even in *beat* null embryos. We quantified the percentage of segments in which the SNb fails to diverge from the ISN either completely (called full bypass), or partially (partial bypass) (Table 1). In *Df(2L)ScoR+18/Df(2L)RM5* embryos, transheterozygous for two overlapping deficiencies that completely remove the *beat* gene (as well as the *Bic-C* gene, see below; Figure 3), 68% of segments show full bypass, and 28% partial bypass. In a control deficiency overlap, *Df(2L)TE116-GW19/Df(2L)RM5*, which removes *Bic-C* but not *beat*, only 5% of segments show full bypass and 2% partial bypass. Transheterozygotes between two *beat* EMS-induced alleles show 38% full bypass, 28% partial bypass. These numbers may suggest that the EMS alleles are hypomorphic for *beat*. Alternatively, the presence of a large hemizygous region in the deficiency overlap may enhance the null condition and account for the low level of bypass phenotypes seen in the $beat^{+}$ deficiency overlap.

beaten path Interacts Genetically With *Fasciclin II* and *connectin*

To test the hypothesis that *beat* encodes an anti-adhesive function, we looked for genetic interactions between *beat* and genes encoding CAMs expressed on motor axons. We reasoned that if *beat* works to oppose adhesion, then a reduction in the amount of adhesion should at least partly compensate for the loss of *beat*. Thus, double mutants between *beat* and CAM genes should have a phenotype less severe than that of *beat* alone. To this end, we created flies carrying various alleles of the *Fasciclin II* (*FasII*) gene and the *beat* gene, being careful to keep the genetic background as nearly identical as possible, and quantified the divergence of the SNb from the ISN using an antibody raised against the LBL protein (Kopczynski et al., 1996) to visualize the motoneurons (Table 2). We used a series of *FasII* mutant alleles that express various levels of the wild-type Fasciclin II protein: $FasII^{e93}$ expresses 100% of the wild-type level, $FasII^{e86}$ expresses ~50%, and $FasII^{e76}$ expresses ~10% (Grenningloh et al., 1991). None of these *FasII* alleles has a major affect on SNb divergence. These *FasII* mutant alleles and wild-type control ($FasII^{e93}$) were generated by imprecise and precise excisions, respectively, of a single P element insert, and thus each genetic interaction is scored in an otherwise identical genetic background. While $FasII^{e93}$; $beat^{2}$ flies have a strong *beat* phenotype with 90% of segments having a divergence defect (n = 116), $FasII^{e76}$; $beat^{2}$ flies have a very significantly more wild-type phenotype, with only 41% of segments having a bypass phenotype (n = 117, $P < 0.00001$). $FasII^{e86}$; $beat^{2}$ flies show a slight but not significant decrease in defects, with 86% of segments having a bypass phenotype (n = 121, P = 0.09), and the defects observed tend to be weaker (see Table 2). This interaction with *FasII* is not allele specific, as $beat^{3}$ is also significantly suppressed by $FasII^{e76}$.

Fas II is expessed on the surface of all motoneurons.

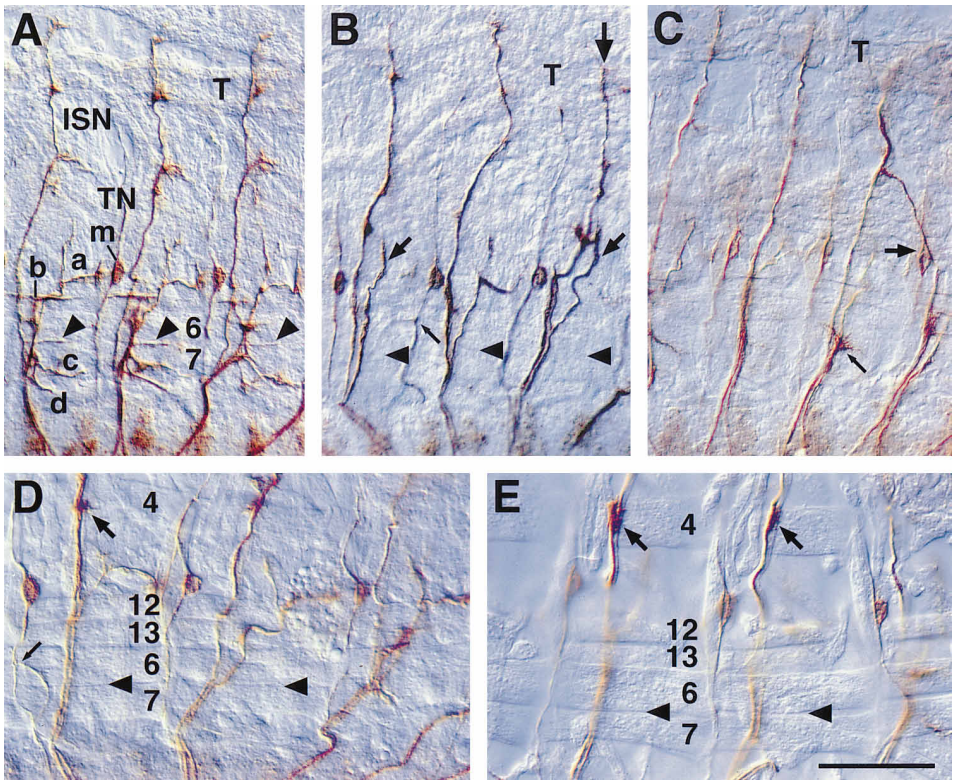


Figure 2. Abnormal Motoneuron Pathways in *beat* Mutants

The anatomy of abdominal segment motor axons pathways in fillet preparations visualized using MAb 1D4, which recognizes Fas II. Anterior is left and dorsal is above. Embryos are stage 17 except (C), which is early stage 16.

(A) The wild-type axon pattern in a *Df(2L)TE116-GW19/Df(2L)RM5* embryo (the SNa, SNb, SNc, and SNd branches are labeled [a], [b], [c], and [d], respectively). The transverse nerve (TN) and m cell (m) lie along the segment boundary. The main tracheal trunk (T) lies in the dorsal region of the bodywall. Arrowheads mark where RP3 innervates the labeled muscles 6 and 7.

(B–E) The *beat* axon pattern in *Df(2L)ScoR+18/Df(2L)RM5* embryos.

(B) Note the lack of nerve branches in the ventral region. The SNa fails to subbranch normally (arrow). The ISN is often trucated at the distal tip (large arrow). The SNb fails to defasciculate with the ISN, leaving muscles 6 and 7 uninnervated by RP3 (arrowheads). The TN is sometimes observed to send processes into the ventral muscle domain in the absence of SNb innervation (small arrow, also in [D]).

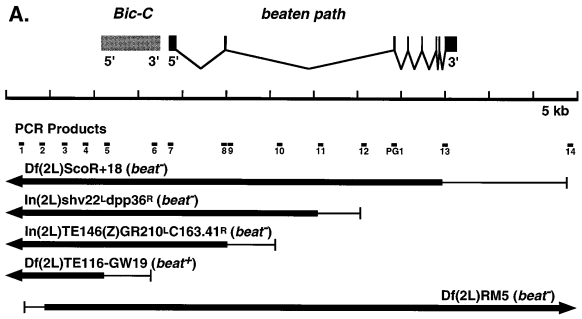
(C) Two ISNs fasciculated across the segment boundary. Note that the posterior ISN remains in contact with the Fas II positive m cell, which channels it into the adjacent segment (arrow). In one segment the SNb, at the choice point, explores the edge of muscle 28 (small arrow).

(D and E) SNb axons remain with the ISN, failing to innervate ventral muscles 6, 7, 12, and 13 (arrowheads), but make ectopic contacts with muscle 4 by lateral sprouting from the ISN (arrows). Scale bar: (A–C) 50 μm, (D–E) 40 μm.

We next asked if *beat* would have a similar genetic interaction with a gene encoding a different CAM that is normally expressed on the surface of only a subset of fasciculating motor axons. The *connectin* (*conn*) gene encodes a leucine-rich-repeat homophilic cell adhesion molecule expressed by SNa and SNc motoneurons, a few ISN motoneurons (that innervate muscles neighboring SNa targets), but no SNb motoneurons (Nose et al., 1992). We used the $conn^{Fvex238}$ allele, which expresses ~5% of the normal level of mRNA (Nose et al., 1994). $conn^{Fvex238}$ has no major effect on divergence of the SNc from the SNa (Table 2). We combined the $conn^{Fvex238}$ allele with the $beat^{2}$ and $beat^{3}$ alleles and scored the percentage of segments in which SNc failed to diverge from SNa. As a control, we scored the percentrage of segments in which SNb failed to diverge from ISN, where *conn* is not expressed and thus unlikely to be involved. We found that while $conn^{Fvex238}$ does not significantly affect SNb divergence from ISN, it does very significantly affect the divergence of the SNc from the SNa. For $beat^{2}$, the percentage of mutant segments drops from 82% ($n = 120$) to 48% ($n = 67$, $P < 0.00001$) in a $conn^{Fvex238}$ background, while for $beat^{3}$ the percentage of mutant segments drops from 77% ($n = 159$) to 44% ($n = 149$, $P < 0.00001$) in a $conn^{Fvex238}$ background. This result indicates that *beat* interacts with multiple cell adhesion systems in a way consistent with it encoding an antiadhesive function.

Mapping and Cloning of the *beaten path* Locus

The *beat* locus was mapped by complementation analysis to the *Adh* region of the 2nd chromsome, between the *Bic-C* and *l(2)35Fa* genes (Ashburner et al., 1990; Mahone et al., 1995). Since the *Bic-C* gene is present on the genomic P1 clone DS00913 (P. Lasko, personal communication), we obtained genomic sequence data for this P1 clone from the Berkeley Drosophila Genome Center. This genomic sequence was analyzed to locate putatively translated regions (see Experimental Procedures). Putatively translated regions were amplified by



B.

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MRFPQNAYQTALTTILLALTLEMTAALRDVRVRVPHAVRRSEKAILKCFY   50
DIEDDSLYSVKWYKGRREFYRYTPKETPPMKVFHFPGVKVRRVSSNESQV  100
VLDAVTMATSGKYSCEVSADAPSFHTLIAAAELEVIETPHNAPFITGIRP  150
RYRVGDILRGNCTSRHSRPAANLTWTVNNEEVNPALVRHHKILRDARNDM  200
ETAVVGIHFVVTDQHFDNGKLKLRCSAQLHDVYWKTTEKIILETDLFPKH  250
GNGANGNHVNPDDFYDQYALHEDHLHNKKNSYLTQLQGEEDDGTEGGLGD  300
GGAAWRGAGSSSSRLSPSSSQSCWMVGGSLMAVLSWTLARQLVSPLQAMM  350
TKTGGATCNMSGRCASRRTRAHGMRVSATKQKQKQRQMQQEHRKSCLRVS  400
KSSWKGRGYVCGCGFGRINSRCIRQLT.                         427
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Figure 3. Molecular Characterization of *beat*

(A) The *beat* exon–intron structure and the locations of deficiency breakpoints in the region. The location of the adjacent *Bic-C* gene is also shown; *Bic-C* is distal to *beat* on the chromosome. This region corresponds approximately to cytological location 35E1-2. The thick lines represent deleted regions and the thin lines represent the region in which the breakpoint lies. PCR primer pairs were used to identify breakpoint regions (numbered). PG1 is the primer pair initially used to identify the *beat* transcript.

(B) The predicted protein sequence of Beat. The potential signal sequence is underlined and N-linked glycosylation sites are in bold.

PCR, then used to synthesize nonradioactive RNA probes to assay the embryonic expression of the regions. One region, termed PG1 (Figure 3A), shows expression in a subset of CNS neurons in positions of known motoneurons (see Figure 4). This PCR fragment was used to probe a Drosophila embryonic cDNA library. Several cDNA clones were isolated, the longest of which is 2.85 kb. Northern analysis of mRNA derived from 6–12 hr embryos shows that these cDNA clones hybridize to a single 3.0 kb transcript.

Sequencing of two cDNA clones, cross-checked with the genomic sequence, predicts that this transcript encodes a novel protein (Figure 3B). Sequence analysis suggests that the first 26 amino acids are a cleaved signal peptide (Von Heijne, 1983). The predicted protein does not contain any other significant hydrophobic stretches, and thus the putative Beat protein should be secreted. There are four possible N-linked glycosylation sites. The predicted protein does not contain any other major protein motifs, nor any stretches of similarity to other proteins. The predicted molecular weight of the protein is 48 kDa, or 44 kDa after cleavage of the signal peptide.

To begin to show that these cDNA clones correspond to the locus that mutates to the *beat* phenotype, we mapped the breakpoints of several deficiency chromosomes that genetically break in or near *beat*. We prepared genomic DNA from flies carrying overlapping deficiencies that remove *Bic-C* and either do or do not remove *beat* (*Bic-C* is homozygous viable; *beat* is semilethal and gives rise to some weak and uncoordinated adults). Utilizing the genomic sequence, we designed PCR primer pairs across the *Bic-C beat* region and assayed the genomic DNA for either absence or presence of PCR product to indicate whether or not the region is removed by the overlapping deficiencies (Figure 3A). This analysis showed that *beat* activity must reside in a 19.6 kb region defined by the breakpoint regions for *Df(2L)TE116-GW19* and *In(2L)TE146(Z)GR210^{L} C163.41^{R}*. This region contains the 5′ end of the putative *beat* transcript. We subsequently showed that this cDNA can be used to rescue the *beat* phenotype (see below).

beaten path mRNA Is Expressed By Motoneurons during Axon Outgrowth

Nonradioactive antisense RNA probes were generated to examine the distribution pattern of *beat* mRNA during embryonic development. No maternally loaded *beat* transcript is present in early embryos. Zygotic expression of *beat* begins in a subset of CNS cells at stage 12/5. While these particular cells could not be unambiguously identified at this stage, based on their positions it is likely that these are early born motoneurons. The number of expressing cells increases during germ band retraction, and by early stage 13 the expression level reaches its maximun. During this period the motoneuron growth cones exit the CNS and begin extending into the periphery. As development proceeds, the number of staining cells appears to remain constant, although cell

Table 1. Motor Nerve Defects in *beaten path* Embryos Are Rescued by *beaten path* Transgenes

	Motor Nerve Branches with Abnormal Morphology					
		SNb			ISN	
Genotype	n	Full Bypass	Partial Bypass	n	Stops Short	Crossed Over
Df(2L)TE116-GW19/Df(2L)RM5 (*Bic-C^{-} beat^{+}*)	83	5%	2%	86	0%	0%
Df(2L)ScoR+18/Df(2L)RM5 (*Bic-C^{-} beat^{-}*)	257	68%	28%	264	65%	4%
beat^{2}/beat^{3}	109	38%	27%	117	40%	3%
C155/+ or Y; beat^{2}/beat^{3}; P[w^{+} UAS-beat]$^{\#1}$/+*	105	4%	3%	105	7%	1%
C155/+ or Y; beat^{2}/beat^{3}; P[w^{+} UAS-beat]$^{\#2}$/+	119	4%	4%	120	3%	0%

* C155 is a GAL4 enhancer trap insert near the *elav* gene, which drives expression in all neurons. Embryos carrying the GAL4 driver and the UAS-beat transgene were selected by staining for panneural Beaten Path protein expression.

Table 2. Suppression of the *beaten path* Phenotype by Mutations in *Fasciclin II* and *connectin*

Genotype	n	SNb		
		Wild Type	Partial Bypass	Full Bypass
FasII^{e93} (100%)*	116	97%	3%	0%
FasII^{e86} (50%)	88	94%	6%	0%
FasII^{e76} (10%)	120	94%	6%	0%
FasII^{e93}; *beat*^{2}	116	10%	51%	39%
FasII^{e86}; *beat*^{2}	121	14%	60%	26%
FasII^{e76}; *beat*^{2}	117	59%	37%	4%

Genotype	n	SNb		n	SNc
		Partial Bypass	Full Bypass		Bypass
conn^{Fvex238} (5%)	119	2%	0%	120	1%
beat^{2}	119	41%	41%	120	82%
beat^{2}; *conn*^{Fvex238}	61	49%	36%	67	48%
beat^{3}	162	39%	45%	159	77%
beat^{3}; *conn*^{Fvex238}	146	37%	51%	149	44%

* The numbers in parentheses show the approximate percentage of wild-type FasII protein and *connectin* mRNA made by the different alleles.

body positions shift due to cell migrations. Counterstaining with antibodies such as MAb 22C10, which stains a subset of CNS neurons including some motoneurons, and MAb BP102, which stains all CNS axon tracts (Figure 4A), indicates that all the identified motoneurons express *beat* mRNA. In addition, the number and positions of the other *beat*-expressing cells are consistent with these cells being the remaining motoneurons (Sink and Whitington, 1991). Thus, *beat* is probably expressed by all motoneurons and exclusively by motoneurons. Expression persists at a high level through stage 14, the period during which the major peripheral motor nerve branches form. After stage 14 the expression level drops to a lower level, then remains constant through stage 17. A small number of cells of unknown function in the embryonic brain also express *beat*. *beat* is not expressed in imaginal discs. Both *beat*^{2} and *beat*^{3} express mRNA normally.

Among the embryonic motoneurons, *beat* mRNA is expressed at different levels. The RP1 and RP3 motoneurons, whose axons leave the ISN in the SNb, express *beat* at very high levels (Figure 4). In contrast, the aCC motoneuron, whose axon pioneers the full length of the ISN, expresses significantly lower levels.

Beat Protein Is Present at Motoneuron Growth Cones

To examine the distribution of Beat protein, we made a Glutathione-S-Transferase fusion protein with a fragment of the Beat protein (amino acids 245–318). Anti-Beat sera from mice recognizes a motoneuron-specific antigen in wild-type embryos (Figure 4B) but not in *beat Df* embryos or in *beat*^{3} mutant embryos. Beat is found around axons and growth cones, especially in choice point regions during the period of branch formation; the appearance of Beat expression is fuzzy in these localized regions, suggesting that Beat is secreted at choice points where motoneurons diverge (Figure 4C). In the *beat*^{2} mutant there is an abnormal accumulation of protein in the cell body, but some protein is found in axons and growth cones. The anti-Beat sera recognizes a single band of ~43 kDa on a Western blot of total protein from 10–15 hr embryos. This is in agreement with the predicted size of 44 kDa for the cleaved, secreted protein.

Panneural Expression of Beat Rescues the Mutant Phenotype

To demonstrate that the *beat* gene encodes the motoneuron antiadhesive function, we made transgenic flies carrying the *beat* cDNA cloned into the pUAST vector (Brand and Perrimon, 1993). The pUAST vector contains the GAL4-responsive upstream activation sequence (UAS) and drives expression only in the presence of GAL4. Embryos carrying the *UAS-beat* construct and the *C155-GAL4* enhancer trap line, which drives GAL4 expression panneurally (Lin and Goodman, 1994), express high levels of *beat* mRNA and protein in all neurons. Stage 17 embryos chromosomally *beat*^{2}/*beat*^{3}, and carrying the *UAS-beat* and *C155-GAL4* constructs, were dissected and scored for the presence or absence of the major motoneuron branches (Table 1). Most segments of these embryos have a wild-type branching pattern. We observed this phenotypic rescue with two independent *UAS-beat* transformant lines.

Embryos carrying *C155-GAL4* and *UAS-beat* express Beat protein on CNS interneurons and on sensory neurons, in addition to motoneurons. We examined whether this ectopic expression of Beat disrupts interneuron and sensory neuron pathways, paying special attention to the Fas II-positive CNS longitudinal pathways (Lin et al., 1994). No defects were observed in these or other CNS or sensory pathways. These results suggest that Beat protein cannot lead to the defasciculation of all axon fascicles that express Fas II, that the *beat* function appears to be highly specific to motoneurons.

Misexpression of Beat on Muscles Causes Motor Axon Guidance Defects

To further assess *beat* function, we crossed the *UAS-beat* lines to the *24B-GAL4* enhancer trap line, which

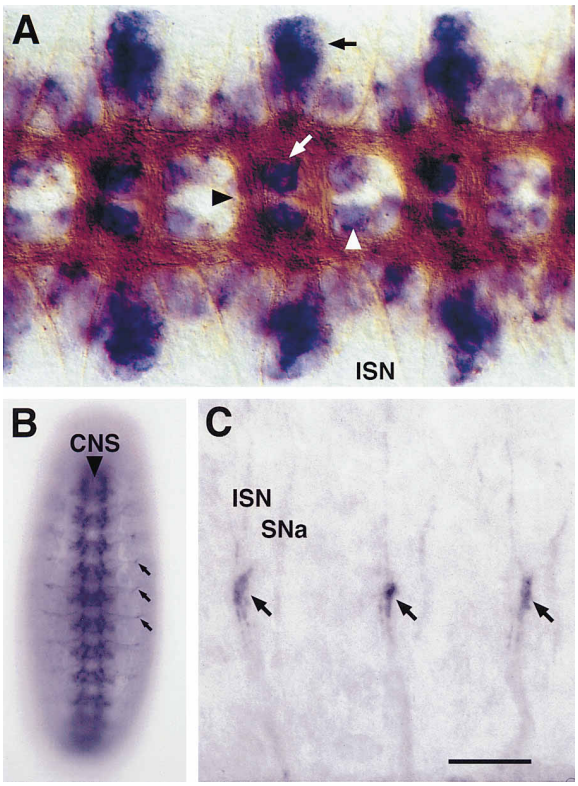

Figure 4. *beat* mRNA and Protein Is Expressed by Motoneurons

(A) Two segments of a stage 14 embryonic CNS stained with MAb BP102 (brown), which stains all CNS axons, and for *beat* mRNA (purple). *beat* is expressed strongly by the RP motoneurons (white arrow), and less strongly by the aCC motoneuron (white arrowhead). The indicated aCC and RP motoneurons project ipsilaterally and contralaterally, respectively (except RP2), into the labeled ISN nerve root. The lateral cluster of cells (black arrow) is in the position of the main motoneuron cluster. The CNS midline is marked with an arrowhead, anterior is to the left.

(B) Whole mount wild-type embryo stained with anti-Beat sera. The same cells stain as in (A). No central axon fascicles are labeled. The arrows mark the ventral choice point region. The labeled arrowhead marks the CNS midline. Anterior is above.

(C) Ventral view of three abdominal segments of a stage 15 embryo in fillet preparation. Diverging growth cones in the ventral choice point region are stained darkly for Beat protein (arrows). The ISN and SNa branches stain less heavily. Anterior is left and dorsal is above. Scale bar: (A and C) 10 μm, (B) 75 μm.

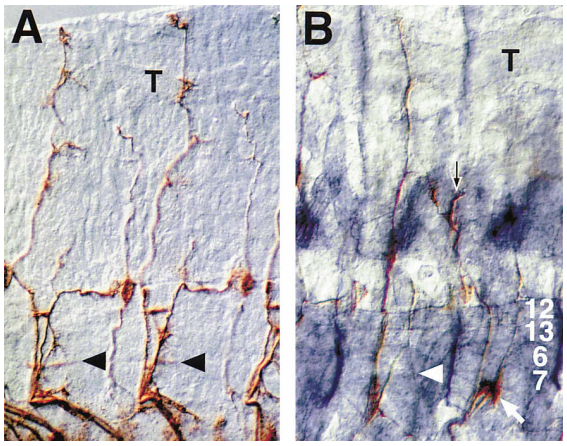

Figure 5. Ectopic Expression of *beat* in Muscles

(A) Two abdominal segments of a wild-type stage 17 embryo in fillet preparation. Arrowheads mark the innervation of muscles 6 and 7 by the RP3 motoneuron. The main tracheal branch is labeled (T). Anterior is left and dorsal is above.

(B) Stage 17 embryo ectopically expressing *beat* in muscles (Beat protein is stained purple). SNb neurons sometimes fail to enter the ventral muscle domain, either bypassing the muscles (left segment) or stalling at the choice point at the edge of muscle 28 (right segment, white arrow). In either case, the ventral muscles are not innervated (arrowhead). Muscle expression also induces ISN pathfinding errors in the dorsal region (black arrow). The ventral longitudinal muscles are numbered.

drives expression in mesoderm (Luo et al., 1994). Homozygous *24B-GAL4; UAS-beat* flies display major motoneuron outgrowth defects. Staining with anti-Beat sera showed that these embryos express high levels of Beat protein in muscles. Control experiments showed that neither *24B-GAL4* alone nor any of the *UAS-beat* lines alone generate any motoneuron phenotype when homozygous, indicating that the motoneuron defects are due to the misexpression of Beat protein on muscles.

Several types of defects were observed in the *24B-GAL4; UAS-beat* embryos (Figure 5B). SNb axons can diverge from the common pathway, but often stall at the edge of muscles 28 and 14. The SNb can also bypass the ventral choice point (Figure 6F). The distal ISN shows abnormal exploration of the dorsal muscle region, sometimes crossing the segment boundary and fasciculating with the ISN from the adjacent segment. Defects were also observed in the other nerve branches. These phenotypes suggest that expression of Beat on muscles disrupts motoneuron–muscle interactions (Figure 6F).

Discussion

In this paper we show that *beaten path* is required for subsets of motor axons to correctly defasciculate from other motor axons at specific choice points, and as a result, motor axons do not properly enter their muscle target regions. This phenotype is 96% penetrant in protein null mutant embryos. The phenotype resembles a result from an earlier experiment where motor axon adhesivity was increased by overexpression of the cell adhesion molecule Fas II (Lin and Goodman, 1994). This similarity suggests that *beat* may normally work to oppose Fas II–mediated adhesivity and allow defasciculation of otherwise adherent sets of motor axons. Genetic analysis supports this interpretation: when motoneurons have reduced quantities of Fas II, they have less need for *beat* function, as evidenced by suppression of the *beat* bypass phenotype double mutant embryos (Figure 6D). This is a dose-dependent interaction, with a 50% reduction of Fas II protein expression having little effect, but a 90% reduction showing a strong suppressive effect. A similar interaction is observed with the *conn* gene (Figure 6E), which encodes another CAM expressed by a subset of motoneurons. In that case, a 95% reduction in *conn* mRNA partially reduces the need for *beat* in those motoneurons that normally expressing *conn* (e.g., SNa) but has no effect on the *beat* bypass

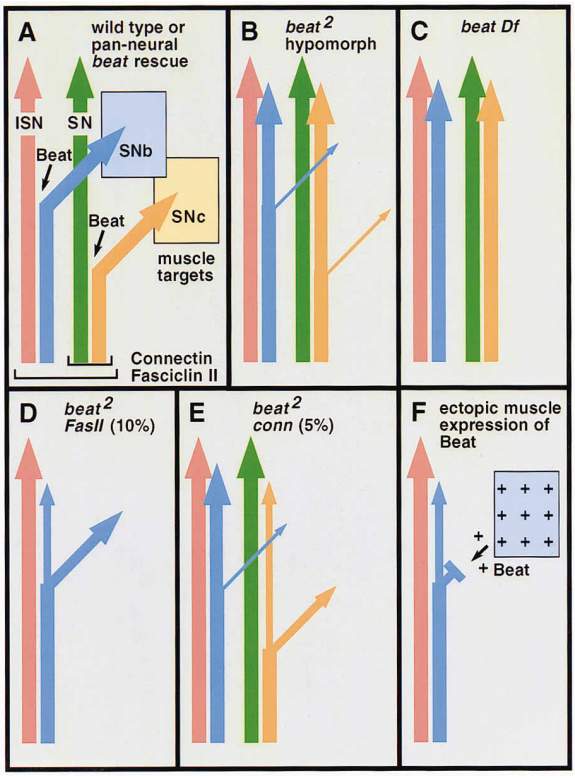

Figure 6. Schematic Diagram of *beat* Function

(A) In wild type embryos, secretion of Beat promotes defasciculation and divergence of motor axon branches at choice points, including the SNb (blue) from the ISN (red), and the SNc (orange) from the SN (green). Fas II is normally expressed on all axons in both the ISN and SN, whereas Connectin is expressed on the axons in the SN.

(B and C) *beat* loss-of-function results in failure to defasciculate at this choice point. The $beat^{2}$ hypomorphic allele (B) displays a less penetrant phenotype than the *beat Df* (C). The relative thickness of the lines after the choice points here and in subsequent panels reflects the percent of segments in which the axons either behave normally or bypass their target region (for numbers, see Tables 1 and 2).

(D) When 90% of Fas II is removed (using the $FasII^{e76}$ allele) in combination with the $beat^{2}$ allele, the bypass phenotype is partially suppressed for the SNb. It is difficult to assay the SNc phenotype in this genotype, since the SNc branch is most easily visualized with the anti-Fas II MAb.

(E) When 95% of Connectin is removed (using the $conn^{Fvex238}$ allele) in combination with the $beat^{2}$ allele, the bypass phenotype is partially suppressed for the SNc (which normally expresses Connectin) but is completely mutant for the SNb (which normally does not express Connectin).

(F) Ectopic expression of Beat by muscles interferes with motoneuron–muscle target interactions, blocking axons from entering their target regions. In some cases the axons stall at the choice point, while in other cases they continue to extend along the ISN or SN, respectively.

phenotype in those motor axons that normally do not express *conn* (e.g., SNb).

beat encodes a novel secreted protein expressed almost exclusively by motoneurons during the period of axon outgrowth. Interestingly, there is some variation in mRNA expression levels in different cells. The aCC neuron, whose axon pioneers the ISN and extends the greatest distance without undergoing defasciculation, expresses *beat* mRNA at much lower levels than do the RP1 and RP3 motoneurons, whose axons defasciculate from the ISN in the SNb branch. This suggests that relative levels of *beat* expression may be important for function.

The *beat* phenotype and its apparent antiadhesive function shed light on the motoneuron outgrowth mechanism. At choice points, motor axons selectively defasciculate from the common pathway and steer into their muscle target region. Several arguments suggest that defasciculation and steering are separable events (Figure 6). First, the *beat* and *side* mutations affect all motoneuron defasciculation events, whereas mutations in genes involved in steering events might be expected to affect subsets of steering decisions. Second, the strong genetic interactions between *beat* and CAM genes, both expressed in motoneurons, are not consistent with a role in steering, which is presumably a motoneuron–target muscle interaction. Finally, motoneurons would be expected to receive steering signals, not send them, and the secreted nature of Beat is not consistent with a receptor role.

The common thread that runs through the phenotypes of the *beat* mutant and the misexpression experiments is that *beat* interferes with cellular adhesive interactions. There is some precedent for motoneurons regulating their ability to interact with each other and their environment. In the chick, motor axons express a PSA-rich form of the neural cell adhesion molecule NCAM in decision regions where they defasciculate from one another (see Introduction). PSA on NCAM appears to regulate cell–cell adhesion by a largely steric mechanism, increasing the distance between apposed cell surfaces (Yang et al., 1992).

Unlike the PSA-rich form of NCAM, Beat is a relatively small and not heavily glycosylated protein (~43 kDa). This suggests that Beat is unlikely to operate by physically separating cell surfaces. Rather it seems more likely that Beat works through a receptor. One candidate receptor is the Fas II protein, with Beat binding to Fas II and blocking Fas II–mediated homophilic adhesion. Several lines of evidence argue against a direct interaction between Beat and Fas II. First, ectopic expression of Beat on Fas II–expressing axon pathways in the CNS does not disrupt axon fasciculation in those pathways. If Beat functions as an antiadhesive for motor axons by directly binding to Fas II, then one would expect that such misexpression of Beat would lead to defasciculation of these Fas II–positive pathways. Second, Beat expression by muscles interferes with motoneuron–muscle interactions, and Fas II has not been strongly implicated in such interactions. Rather, the simplest interpretation of this result is that Beat interferes with specific interactions of motor axons with their target muscles, events that do not normally involve Fas II. Third, there exists a second gene, *side*, that mutates to an identical phenotype as *beat* and thus is a candidate gene to encode the *beat* receptor. A molecular analysis of *side* is in progress. Finally, cell coaggregation experiments using S2 cells expressing Fas II, and S2 cells expressing Beat tethered to the membrane with a GPI anchor, do not show any evidence of a binding interaction between Beat and Fas II (D. F. and C. S. G., unpublished data).

How might Beat promote defasciculation? If Beat works through a heterologous receptor that is not Fas II, then it is likely to operate by a cytoplasmic signaling mechanism. First, Beat might lead to the modification of CAM binding function. Alternatively, CAMs also operate as signaling molecules (reviewed by Doherty and Walsh, 1994), and thus Beat might regulate the connection of CAMs to the cytoskeleton or to other signaling events (Figure 6). Distinguishing among these alternatives awaits identification of the Beat receptor.

Another possibility is that Beat might act to cause the internalization of CAMs. In Aplysia, treatment of sensory neurons with 5-HT results in the internalization and degradation of apCAM, the structural homolog of Fas II and NCAM (Bailey et al., 1992; Mayford et al., 1992). The reduction in surface apCAM results in decreased fasciculation and increased sprouting. Our data does not directly address whether such a mechanism may be operating in Drosophila. However, we do not observe an increase in Fas II immunoreactivity in *beat* mutants, nor a decrease in immunoreactivity when *beat* is panneurally expressed in a wild-type background.

Fas II, NCAM, and apCAM are all closely related members in the Ig superfamily. Thus far, at least three distinct mechanisms have been discovered that appear to be used to regulate the adhesion mediated by these closely related CAMs. apCAM can be regulated by internalization and degradation in response to transmitters. NCAM can be regulated by addition of a large carbohydrate moiety—PSA—with general effects on cell interactions. And as shown in this paper, Fas II can be regulated through the action of a small secreted protein—Beat. It remains to be determined why these different mechanisms of CAM regulation are used in different contexts. One thing seems clear from all of these studies: cell adhesion is tightly regulated in the nervous system, and changes in the level of adhesivity can have profound effects on the ability of axons to selectively fasciculate and defasciculate.

Experimental Procedures

Genetics and Fly Stocks

The *beat*^{2} and *beat*^{3} mutations were introduced with 25 mM EMS in a *FasIII*^{E25} mutant background, as descibed previously (Van Vactor et al., 1993). The phenotypes described in this paper do not require the *FasIII*^{E25} background. Transgenic fly stocks were generated by ligating an ApoI/DraI fragment containing the *beat* coding region into the pUAST vector (Brand and Perrimon, 1993).

Molecular Analysis

Genomic sequence data for P1 clone DS00913 was obtained from the Berkeley Drosophila Genome Center. The sequences were assembled with Lasergene software (DNASTAR) and analyzed using a Drosophila-specific version of GRAIL (Xu et al., 1995) and a version of Genefinder (P. Green and L. Hillier, personal communication) customized for Drosophila (G. Helt, unpublished data). PCR primer pairs were designed to amplify predicted translated regions, and used to generate RNA probes for in situ hybridization (C. Kopczynski and C. S. G., unpublished data).

Deficiency breakpoints were mapped by the presence or absence of specific PCR amplification products. Using adult flies transheterozygous for overlapping deficiencies, genomic DNA was prepared and assayed with a series of primer pairs (see Figure 3A; pairs 13 and 14 are STS Dm0023 and Dm0044, respectively). Primer pair 5 served as a negative control, as it was known to be removed by both *Df(2L)TE116-GW19* and *Df(2L)RM5* (Mahone et al., 1995).

beat cDNAs were isolated from a 9–12 hr cDNA library (Zinn et al., 1988) using the PG1 PCR product as a probe. Sequencing reactions were analyzed on an ALF sequencer (Pharmacia) and assembly was performed on Lasergene software.

Antibody Production

A BglII/SacI fragment of the *beat* cDNA (amino acids 245–318) was cloned into the BamHI/SacI sites of the pGEXbubba1 vector (Gerry Rubin lab). Fusion proteins were purified from expressing bacteria using glutathione-agarose beads. For immunization of mice, ~150 μg of protein was emulsified in RIBI adjuvant (Immunochem Research) and injected at 2-week intervals. Prior to use, sera were preincubated against 0–5 hr embryos, which do not show any detectable *beat* transcript.

Histology

Histology of the embryonic nervous system was performed as described (Van Vactor et al., 1993), except with anti-Beat, which was diluted 1:100 and 100 mM maleic acid 150 mM NaCl (pH 7.5) was substituted for PBS and 1% blocking reagent (Boehringer Mannheim) was substituted for NGS.

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